



Europäisches Patentamt

(19)

European Patent Office

Office européen des brevets



(11)

EP 0 523 949 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
25.06.2003 Bulletin 2003/26

(51) Int Cl.7: C12N 15/13, C07K 16/18,
A61K 39/395, C07K 16/00

(21) Application number: 92306420.8

(22) Date of filing: 14.07.1992

(54) Production of antibodies

Herstellung von Antikörpern

Production d'anticorps

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU MC NL
PT SE

(30) Priority: 15.07.1991 GB 9115284
01.08.1991 GB 9116594
23.03.1992 GB 9206284

(43) Date of publication of application:
20.01.1993 Bulletin 1993/03

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Description

[0001] This invention concerns the production of recombinant primate antibodies by DNA technology, their expression in eukaryotic cell lines and the use of such antibodies in the therapeutic and prophylactic treatment of human beings.

5 [0002] Antibodies, or immunoglobulins, are proteinaceous bifunctional molecules. One region, which is highly variable between different antibodies, is responsible for binding to an antigen, for example, the many different infectious agents that the body may encounter, whilst the second, constant region is responsible for binding to the Fc receptors of cells and also activates complement, a complex system of proteins responsible for cell lysis. In this way, antibodies represent a vital component of the immune response of mammals in destroying foreign microorganisms and viruses.

10 [0003] Antibodies are divided into different classes on the basis of the structure of the constant region. In humans for example, five major structural classes can be identified immunoglobulin G or IgG, IgM, IgA, IgD and IgE. Each class is distinguished on the basis of its physical and biological characteristics which relate to the function of the immunoglobulin in the immune system. IgGs can be further divided into four subclasses: IgG1, IgG2, IgG3 and IgG4, based on differences in the heavy chain amino acid composition and in disulphide bridging (see below for explanation), giving rise to differences in biological behaviour. A description of the classes and subclasses is set out in "Essential Immunology" by Ivan Roitt, Blackwell Scientific Publications.

15 [0004] An antibody molecule is composed of two light chains and two heavy chains that are held together by interchain disulphide bonds. Each light chain is linked to a heavy chain by disulphide bonds and the two heavy chains are linked to each other by disulphide bonds. Each heavy chain has at one end a variable domain followed by a number of constant domains, and each light chain has a variable domain at one end and a constant domain at the other end. The light chain variable domain is aligned with the variable domain of the heavy chain. The light chain constant domain is aligned with the first constant domain of the heavy chain. The remaining constant domains of the heavy chains are aligned with each other. The constant domains in the light and heavy chains are not involved directly in binding the antibody to the antigen.

20 [0005] The variable domains of each pair of light and heavy chains form the antigen binding site. They have the same general structure with each domain comprising a framework of four regions, whose sequences are relatively conserved, connected by three complementarity determining regions (CDRs). The four framework regions largely adopt a beta-sheet conformation and the CDRs form loops connecting, and in some cases comprising part of, the beta-sheet structure. The CDRs are held in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site.

25 [0006] The antibody chains are encoded by genes at three separate loci on different chromosomes. One locus encodes the heavy chain isotypes and there are separate loci for the κ and λ light chains, although a B-lymphocyte only transcribes from one of these light chain loci. The genes which encode antibody variable domains are generated during B-lymphocyte ontogeny by a process of recombination involving the joining of the V, D and J gene regions. A single B-lymphocyte will only use one heavy chain and one light chain recombined variable domain to ensure that it only has one antigen specificity. So only one allele of the heavy chain and one allele of the light chain are expressed by a single B-lymphocyte. This is known as allelic exclusion.

30 [0007] The exposure of an animal to an antigen by infection or immunisation, results in the production of different antibodies with different specificities and affinities. An antiserum obtained from the immunised animal will, therefore, be heterogeneous and contain a pool of antibodies produced by many different lymphocyte clones. Antibodies thus obtained are referred to as polyclonal antibodies and this polyclonal nature has been a major drawback in the use of antibodies in therapeutic applications and in diagnostic assays.

35 [0008] A major step forward occurred in 1975 when Kohler and Milstein (*Nature*, 1975, 256, 495-497) reported the successful fusion of spleen cells from mice immunized with an antigen with cells of a murine myeloma line. The resulting hybrid cells, termed hybridomas, have the properties of antibody production derived from spleen cells and of continuous growth derived from the myeloma cells. Each hybridoma synthesizes and secretes a single antibody to a particular determinant of the original antigen. To ensure that all cells in a culture are identical, i.e. that they contain the genetic information required for the synthesis of a unique antibody species, the hybridomas resulting from cell fusion are cloned and subcloned. In this way, the cloned hybridomas produce homogeneous antibodies of the original animal species from which the spleen cells were derived.

40 [0009] The ability to produce monoclonal antibodies has revolutionised the diagnosis of many diseases and provides the possibility of prevention and immunotherapy of numerous pathological disorders. Unfortunately, foreign antibodies namely antibodies of non-human species, such as a mouse or a rat, administered repeatedly to a human for vaccination or treatment, will be recognised by the individual's immune system and are likely to cause an undesirable anti-globulin response. This anti-antibody response is due to the foreign origin of the constant domains and the four framework regions. The result of this response is likely to be the neutralising of the therapeutic antibody, and the triggering of harmful anaphylactic or allergic reactions. Furthermore, non-human monoclonal antibodies do not fix human complement particularly well and are less likely to trigger non-specific mechanisms of cell clearance such as antibody - de-

pendent cell - mediated cytotoxicity (ADCC), in view of the differences in effector function of the constant region of the antibody. Non-human monoclonals are therefore not as effective as human antibodies in clearing infected or diseased cells. And so, for a therapeutic antibody to be effective and to remain in the circulation without raising an anti-antibody response, it must be able to escape recognition by the recipient's immune system.

5 [0010] One solution to this problem is to form chimeric antibodies as described in Morrison *et al* (P.N.A.S., 1984, 81, 6851-6855); and Neuberger *et al* (Nature, 1985, 314, 268-270), where the variable region of a foreign species derived from a hybridoma as described above, is grafted to the constant region of human antibody. However, in this situation the variable region remains foreign to the recipient; thus, it may be recognised and may still pose a significant immunogenicity problem. The humanisation of an antibody, as described in Jones *et al* (Nature, 1986, 321 522-525); and Riechmann *et al* (Nature 1988, 332, 323-327), in which the CDRs of a foreign antibody species are grafted onto a human antibody framework, does alleviate many of these problems. However, the CDR-grafting of antibodies is a complicated process and the resultant antibody may require further modification to maintain its binding affinity. It is therefore clear that to avoid recognition by the human immune system, the optimum form of antibody is a human antibody or an antibody which is substantially identical to a human antibody.

15 [0011] Anecdotal reports have suggested that apes (e.g. chimpanzees) and monkeys (e.g. cynomolgus, rhesus, aotus) which are commonly used as laboratory animals, have immune responses sufficiently close to those of humans to provide good models of infection. It has also been suggested that their antibodies may be sufficiently homologous to human antibodies to overcome some of the problems faced with, for example, rodent antibodies. There is no published evidence of this and few if any non-human primate cell lines are available for testing. The process of obtaining such antibodies is therefore exceedingly problematic. Cell lines have been produced from human lymphocytes but such lines have low stability, do not readily form hybrids which might be more stable, and usually produce low yields of antibodies when cultured *in vitro*. Primate cells may also harbour foreign infectious nucleic acid, for example, from a virus, which poses problems of cross-infection of the human to be treated. Lengthy purification and/or sterilisation procedures must be applied before the antibody produced therefrom is in an acceptable form for administration to humans.

25 [0012] European patent Publication No. 314161 discloses a process for the production of human immunoglobulin in a eukaryotic host cell. The host cell is transfected with operably linked first and second genes which code for human heavy chain variable and constant regions respectively. The host cell is also transfected with operably linked genes coding for variable and constant regions of a human light chain. This transfected cell is cultured and recombinant human immunoglobulins, having variable regions of the desired binding specificity, can be recovered from the cell culture. However, this process poses a number of problems: i) a large quantity of lymphocytes is required for recovery of sufficient genomic DNA to carry out the process. The specification discloses, the removal of $1-2 \times 10^8$ lymphocytes from which 121 μg of genomic DNA were retrieved; ii) all four alleles (two for the heavy chain and two for the light chain) are retrieved in the genomic DNA and require extensive sequencing and selection by cloning, expression and binding studies, to obtain the functional pairing of genes (one for heavy chain and one for the light chain) for further processing; 35 iii) poor expression levels of antibody are achieved by culturing the host cells transfected by the described process. The specification discloses yields of 6.7 - 34.5 $\mu\text{g}/\text{ml}$ which average out at 20 $\mu\text{g}/\text{ml}$, and using a construct including a cytomegalovirus (CMV) expression enhancer only 1 $\mu\text{g}/\text{ml}$.

40 [0013] International Publication WO91/04336 also discloses a process for the production of human monoclonals through the recovery of genomic DNA.

[0014] It is quite clear that this process suffers from the same disadvantages described above for EP314161.

[0015] The advent of PCR has allowed the generation of cDNA clones from mRNA derived from small numbers of cells. Although PCR remains a very powerful tool, the requirement for knowledge of the sequences of the 5' and 3' end of the target cDNA sequence hampers the use of this technique for the generation of cDNA clones from mRNA coding for proteins with varied sequences, especially secreted products with diverse leader sequences such as the human antibody family. Although many human H chain sequences have been reported [Kabat E.A. *et al*. Sequences of Proteins of Immunological Interest, 4th Ed, US Dept. of Health and Human Services, US Govt. Printing Service 1987], and the PCR primers are designed from the consensus sequences of these, not all human V regions will be amplified using these primers due to the 3' bases being incompatible to elongation by Taq polymerase.

50 [0016] Larrick *et al*, Biotechnology, 7(9), 1989 : 934-938 and Levy *et al*, Gene 54 (2-3) 1987 : 167-173 disclose the use of cDNA for encoding only the variable region of antibodies but not the constant region. The present invention in contrast provides a new process involving conventional recombinant cDNA cloning technology to facilitate the rescue of complete (i.e. variable and constant) human, heavy and light chain antibody genes and their expression in eukaryotic cells using high level eukaryotic expression vectors for the immortalisation of functional antibodies.

55 [0017] Furthermore, the inventors have been able to demonstrate that cDNA cloned from a non-human primate peripheral blood lymphocyte and the antibody chain produced therefrom, does in fact show sufficient homology to a human antibody chain sequence to provide potentially useful therapeutic agents. The invention therefore includes a process for the rescue of non-human primate heavy and light chain antibody genes and their expression as described

above.

[0018] The invention therefore provides a process for the production of a recombinant primate antibody comprising:

- (i) selecting a primate lymphocyte-derived cell line that is capable of expressing a desired antibody;
- (ii) isolating RNA from the cell line and separating mRNA from the other RNAs so isolated;
- (iii) synthesising cDNA from the mRNA and inserting the cDNA into a cloning vector;
- (iv) transforming a host cell with the vector containing the cDNA to obtain a library;
- (v) screening the library for cDNA encoding the antibody heavy and light chain genes;
- (vi) inserting the cDNA encoding the genes into an expression vector;
- (vii) transfecting a host cell with the expression vector containing the cDNA; and
- (viii) culturing the transfected host cell and isolating the desired antibody.

[0019] The term 'primate' is taken to mean prosimians (e.g. Lemurs), new world monkeys (e.g. aotus), old world monkeys (e.g. cynomolgus), apes (e.g. chimpanzees) and humans.

[0020] Reference to a primate lymphocyte-derived cell line, means a cell line derived from a single primate lymphocyte which will produce a single antibody. The cell line must be sufficiently stable to enable recovery of RNA and so is preferably stabilised or immortalised using conventional viral transformation and/or hybridoma technology (as described in Methods of Hybridoma Transformation, Barta and Hirsaut (eds), Humana Press, Clifton, N.H. 1985). Such cell lines may be obtained from depositories such as the American Type Culture Collection of Rockville MD, USA.

[0021] The cell line may be produced by removing lymphocytes, namely lymphoblastoid cells or, B-lymphocytes, from the peripheral blood lymph nodes or from the spleen for example from an individual (human or non-human primate) known to have recovered from or be in remission from a disease state; from an individual known to be infected with a pathogenic organism or suffering from cancer or an autoimmune disease but who does not manifest full disease symptoms; from an individual who has been vaccinated or inoculated with antigen and has mounted an antibody response; from healthy individuals followed by screening for useful antibody. Examples of disease states include an infection by a pathogenic organism (eg. virus or bacteria), wherein removal of lymphocytes preferably takes place within two to three months post recovery or during the phase of high antibody titre, or an individual who has received vaccination against an antigen for example of a pathogenic organism, wherein removal of lymphocytes preferably takes place within two to three months post immunisation. Individuals in whom a pathogenic organism such as a virus can be detected but who do not progress to a full disease state, may also provide an extremely useful source of antibody-producing cells. Clearly, it is possible when using non-human primates to inoculate with a pathogenic organism rather than an attenuated form of the organism which is frequently the form used for vaccination of humans. The immune response to this organism may well be far greater, thus providing a better source of antibody-producing cells, than from the vaccinated individual.

[0022] A cell line may also be produced by removing lymphocytes from an individual who is known to be suffering from a disease state such as cancer or an autoimmune disease and can be demonstrated to be making an antibody response directed against either tumor cells or self antigens. Lymphocytes may also be obtained from an individual who is identified as showing spontaneous remission of their cancer or who have been vaccinated with tumor antigens, and have mounted an antibody response. An alternative approach for the identification of useful lymphocytes involves screening cohorts of individuals known to be at risk of developing cancer through exposure to certain environmental factors or through a genetic predisposition for example "cancer families" which display a greater than average occurrence of cancer within the population. These cohorts may be screened in particular, for individuals who do not develop cancer as a result of their ability to raise antibody to the tumor antigens. It may also be possible to screen healthy individuals for anti-tumor antibodies based on a theory that cancerous cells are present in all individuals, the immune system is generally able to mount a response namely by the production of antibodies, to remove the mutated cells before a cancerous state is reached. If this is so, useful lymphocytes may be obtainable from any individual. The lymphocytes so identified may then be stabilised by viral transformation and/or fusion as described below.

[0023] Viral transformation is preferably carried out using Epstein Barr Virus (EBV). Most peripheral blood B-lymphocytes have a receptor for EBV and when infected by the virus these cells are transformed with the accompanying expression of the EBV nuclear antigen (EBNA). However, only around 20% of cells are "immortalised" *in vitro* and these are in general only small non-activated B cells. Plasma cells (activated B-cells) lack the EBV receptor so resistance to EBV infection appears to increase with maturity reducing the effectivity of viral transformation by this route when the recovered lymphocytes are mature.

[0024] For viral transformation using EBV it is therefore preferable to use non-plasma cell peripheral blood lymphocytes. To establish a cell line, supernatant from a cell line producing the virus such as B95.8 (Miller et al 1972 Proc. Natl. Acad. Sci. USA 69 383-387) generally contains sufficient infectious virus particles. In practice pellets of up to 10⁷ cells are suspended in approximately 1ml of the viral culture supernatant and incubated at 37°C for about 1 hour. This allows attachment of the virus to specific receptors on B-cells and cell penetration. It is preferable to agitate the container

gently to prevent sedimentation. The cells so infected can then be cultured and the genes for the desired antibody can be cloned.

[0025] An alternative or an additional step to viral transformation is to fuse to myeloma cells to provide stabilisation (Crawford, D.H. 1985 Human Hybridomas and Monoclonal Antibodies Ed. E.G. Engelman, S.K.H. Fong, J. Larrick and A. Raubitschek pp 37-50 or Roder J.C. *et al* The Epstein-Barr virus-hybridoma technique *ibid* pp55-67). The myeloma is optionally a heterohybridoma preferably of mouse/human origin. A suitable heterohybridoma can be generated for example from an antibody secreting cell-line such as HT01. Suitable cells can be selected on the basis of their sensitivity to hypoxanthine aminopterin and thymidine by subjecting them to sequential passage through medium containing 8-azaguanine as they are aminopterin sensitive.

[0026] In order to use such a heterohybridoma, the genes encoding endogenous human heavy and light chains must be deleted, otherwise the final cell line will be capable of producing more than one antibody and will not contain heavy and light chains for the desired antibody alone. This can be achieved by subjecting the cells to a 90% lethal dose of ultra-violet irradiation and selecting for suitable colonies by cytoplasmic staining with anti-human Ig and chromosome number namely polyploid with between 60-140 mouse chromosomes.

[0027] It is also advantageous to select vigorously growing cells. This may be achieved by passaging through the peritoneal cavity of a 2,6,10,14-tetramethylpentadecane (or pristane) primed mouse.

[0028] Final selection for growth, karyotype and fusability is then carried out, karyotype being the most important. The ideal heterohybridoma contains mainly mouse chromosomes.

[0029] Selection of a target lymphocyte cell line may be carried out by screening for the production of antibody which has affinity to the desired antigen, and antibody functionality.

[0030] Testing for affinity can be achieved by immunoassay techniques for example radioimmunoassay or Enzyme Linked Immunosorbent Assay (ELISA). Immunoassay techniques such as these use the specific interaction of antibody with antigen to provide information about antigenic specificity. Radioimmunoassays assess antibody level either by determining the capacity of antibody to complex with radioactive antigen or by measuring the amount of antibody binding to an insoluble antigen preparation. The ELISA technique involves conjugating enzymes to antigens or antibodies. The enzymes are usually selected on the basis of simple kinetics and can be measured by a coloured reaction product for example by spectrophotometry. Preferred enzymes include alkaline phosphatase, β -D-galactosidase and horseradish peroxidase. ELISA can be employed as a primary binding or a competitive binding assay. For example, lymphocytes isolated from an individual infected by a virus can be selected by culturing supernatant medium from one lymphocyte cell line in the presence of a suitably labelled viral antigen possibly in the form of whole or empty viral particles. The antibody/antigen complexes can then be identified as described above and the respective lymphocyte cell line selected for further studies.

[0031] The test for functionality of the antibody in the case of an infectious agent may involve competition studies for neutralisation for example viral neutralisation. Neutralisation studies can be carried out using Radioimmunofocussing Assay (RIFA), in which a fixed concentrate of purified antibody is cultured with equal volume of 10 fold dilutions of virus and then assayed for virus titre. An alternative test can be to use complement and test for cell lysis.

[0032] It is possible to obtain cells secreting human/or non-human primate IgG, IgG1, IgG2 or IgG3 IgM, IgA, IgD or IgE. These can be selected by Ouchtolony agar double diffusion or ELISA.

[0033] Following selection of a lymphocyte cell line expressing a functional antibody with the desired specificity, the total cell RNA can be isolated using standard recombinant techniques such as the method of Chomczynski and Sacchi (1987, *Anal. Biochem.* 162, 156-159). In order to isolate the specific messenger RNA (mRNA) encoding the antibody, standard techniques are also employed for example as described in *Molecular Cloning: A Laboratory Manual* by Maniatis *et al* Cold Spring Harbor Laboratory Press.

[0034] Complementary DNA (cDNA) is then synthesised from the mRNA by standard recombinant techniques as for example disclosed in the aforementioned *Molecular Cloning Manual*.

[0035] It is possible to employ between 1.3×10^4 and 1.3×10^7 lymphocytes for isolation of total cell RNA, however the invention further provides a method of cloning full length antibody genes from a much smaller number of cells as would be required from unstable antibody producing hybridomas or EBV transformed B cells of unknown stability. This method is therefore particularly useful for rescuing human or non-human primate antibodies from unstable cell lines, but may be applied to the rescue of any antibody heavy and/or light chain genes. This is made possible by advances in the reproducibility and quality of commercially available enzymes and vector systems for conventional cDNA cloning and the introduction of micro-RNA isolation techniques.

[0036] This improved method can be achieved by generating a size-selected cDNA library from as few as 1000 hybridoma cells. This library or a fraction of it may then be screened for immunoglobulin chains.

[0037] The invention therefore provides a method of cloning full length antibody genes comprising i) micro-RNA preparation from approximately 1000 cells, ii) generation of a size-selected cDNA library iii) screening the library for cDNA encoding heavy and light chains and iv) isolating the cDNA encoding the heavy and light chains.

[0038] The invention also provides a process for the production of a recombinant antibody comprising:

- i) micro-RNA preparation from approximately 1000 cells;
- ii) generation of a size-selected cDNA library;
- iii) screening the library for cDNA encoding the heavy and light chains and isolating the same;
- iv) inserting the cDNA encoding the heavy and light chains into an expression vector;
- v) transfecting a host cell with the expression vector containing the cDNA; and
- vi) culturing the transfected host cell and isolating the desired antibody.

[0039] Identification of cDNA clones which encode the antibody heavy and light chain proteins can be achieved by cloning the cDNA into a replicable vector for example a plasmid, and transforming a host cell for example a prokaryote such as *E. coli*. The resultant library can then be screened for antibody light and heavy chain cDNA in the following manner.

[0040] Screening can be carried out using heavy and light chain DNA probes with detectable labels and a detection method for example as described in Gene Cloning by D.M. Glover (Published by Chapman and Hall Ltd London). These techniques can involve radiolabelling and detection by radiography methods or non-radioactive labels for example digoxigenin 11 dUTP and a detection kit for example the Nonradioactive DNA labelling and Detection kit available from Boehringer Mannheim. This screening method also allows completely unclassified antibodies to be screened for isotype using mixed probes for example human γ , μ and α H chain probes or κ and λ L chain probes.

[0041] Clones can be selected and if desired the sequence of the antibody heavy and light chains can be determined. It is also possible to introduce modifications into the antibody cDNA at this stage prior to preparation of vectors for expression; this may involve single codon or whole region modification. For example, class or species switching of the antibody isotype can be undertaken (ie: to form chimaeric antibodies). This can be achieved by generating fusions of the isolated V regions with the cDNA from the isotype of choice.

[0042] Once a suitable cell colony has been selected, the cDNA sequences for the light and heavy chain genes can be subcloned into vectors suitable for insertion into a host cell for expression. Construction of the expression vectors may be carried out in accordance with procedures known in the art (Molecular Cloning: A Laboratory Manual, Second Edition, Maniatis *et al*, Cold Spring Harbor).

[0043] The host cell must be capable of expressing the antibody in a functional form and therefore, should be a eukaryotic cell such as a mammalian cell e.g. myeloma or chinese hamster ovary (CHO) cells which are capable of carrying out post-translational modifications, in particular, correct folding of the chains, and glycosylation, which can be essential for effective functionality of the constant region of an antibody. Eukaryotic cells can be cultured in vitro quite successfully and are known to express functional antibody. Yeast or insect cells may also serve as host cells as they can also carry out desired post-translational modifications.

[0044] The heavy and light chain cDNA can be transfected in a single vector as described in WO87/04462 or co-transfected in two vectors as described below. Reference hereinbefore and hereinafter to transfection of a host cell line with an expression vector includes within its meaning co-transfection of the host cell employing more than one vector unless it is clear from the context that only co-transfection is being referred to.

[0045] The vectors for co-transfection preferably contain independently selectable markers, the resulting colonies may thus be selected for both markers. Colonies exhibiting the dual phenotype are generally capable of co-expressing both the light and heavy chains. The selectable markers may or may not be of a dominant nature. Examples of selectable markers include adenosine deaminase (Kaufman *et al*, *P.N.A.S.*, 1989, 83 3136-40) asparagine synthetase (Cartier *et al*, *Mol.Cell Biol.*, 1987, 7, 1623-28), *E.coli* trpB gene and *Salmonella* hisD gene (Hartman *et al*, *P.N.A.S.*, 1988, 85, 8407-51), M2 mouse ribonucleotide reductase (Thelander *et al*, *EMBO J*, 1989, 8, 2475-79), human multidrug resistance gene (Kane *et al*, *Gene*, 1989, 84, 439-446), glutamine synthetase (Bebbington *et al*, *DNA Cloning*, Vol III, 1987, Ed. D.M. Glover, 163-188, IRL Press), xanthine guanine phosphoribosyl transferase (gpt) (Mulligan *et al*, *Science*, 1980, 209, 1422-27), hygromycin B (Santerre *et al*, *Gene*, 1984, 30, 147-156), neomycin gene (Southern *et al*, *J. Mol. Appl. Genet.*, 1982, 1, 327 341), and dihydrofolate reductase (Subramani *et al*, *Mol.Cell Biol.*, 1981, 1, 854-868).

[0046] A preferred selectable marker for use with one of the vectors is dhfr which is usually employed with a parental Chinese Hamster Ovary (CHO) cell line of the dhfr phenotype (Urlaub *et al*, *P.N.A.S.*, 1980, 77, 4216-4220). Successfully transfected CHO cells will possess the dhfr⁺ phenotype and can readily be selected by culturing the colonies on media devoid of thymidine and hypoxanthine and optionally containing methotrexate (MTX). A preferred selectable marker for use with the other of the vectors is a dominant resistance marker, such as neomycin (neo). CHO cells successfully transfected with this marker can readily be selected by culturing the colonies in media containing the antibiotic, Geneticin, an analogue of neomycin.

[0047] Another expression system for use with CHO or myeloma cells is the glutamine synthetase (GS) amplification system described in WO87/04462 the method of which is incorporated herein by reference. This system involves the transfection of a glutamine dependent cell with a gene encoding the GS enzyme and the desired antibody heavy and light chain genes. Cells are then selected which grow in glutamine free medium. These selected clones are then subjected to inhibition of the GS enzyme using glutathione sulfoximine (Msx). The cells, in order to survive, will amplify

the GS gene with concomitant amplification of the gene encoding the antibody.

[0048] As described previously a selectable marker such as GS preferably also provides the basis upon which the genes encoding the light and heavy chains may be amplified. In transfection of a cell line, the vector DNAs are often integrated into the chromosome of the cell at the same locus. Thus, the use of a selectable marker as the basis for amplification normally results in a parallel increase in the copy number of both genes. Similarly, dhfr is a selectable marker which enables desired amplification through the use of increasing concentrations of the inhibitor MTX.

[0049] The selectable markers are of course under the control of regulatory elements of DNA so as to provide for their expression. The regulatory elements are preferably of a viral source, such as from DNA tumour viruses. Particularly preferred are the SV40 or adenovirus major late promoter. It is particularly advantageous in this regard to remove the enhancer element from the promoter thus effectively "crippling" it. This modification allows for increased levels of gene amplification at each concentration of inhibitor than would otherwise occur if a strong promoter was used. In the case of the use of GS as a selectable marker, an example of a suitable promoter is the mouse metallothionein promoter or preferably the human cytomegalovirus (hCMV)-MIE promoter described in PCT patent publication number WO89/01036.

[0050] The antibody light and heavy genes are also under the control of regulatory elements of DNA so as to provide for their expression. The use of the same regulatory elements for both chains is preferred so that their expression is substantially balanced. The regulatory elements may be of viral origin and examples include those mentioned above in conjunction with the expression of dhfr or GS as a selectable marker. Another example is the use of the β -actin promoter and cognate β -actin polyadenylation signal.

[0051] One or both of the vectors may also contain an SV40 origin of replication to allow for the vector constructs to be checked by rapid transient assay for example in COS cells.

[0052] Co-transfection of the cell line with the expression vectors may be carried out simply by using equimolar quantities of both vectors and standard transfection procedures, such as calcium phosphate precipitation or lipofectin. Selection of the desired co-transfected cell line may be carried out in accordance with standard procedures known for the particular selectable markers.

[0053] The invention therefore includes a vector suitable for transfection of a host cell comprising cDNA encoding primate antibody heavy and light chains. The invention therefore includes a eukaryotic cell line transfected with cDNA for the expression of primate antibody heavy and light chains.

[0054] The invention further includes a process for the expression of cDNA encoding primate antibody heavy and light chains comprising transfecting a eukaryotic host cell with a vector or vectors suitable for the expression of said cDNA.

[0055] Culture of the cell line may be carried out in serum-containing or preferably serum-free media. It is particularly advantageous during purification if protein-free medium is employed. Where the cell line is a CHO dhfr⁺ transformant, the medium preferably lacks hypoxanthine and thymidine and optionally contains MTX. When using the GS system it is advantageous to employ a glutamine dependent cell line and a glutamine free medium. Expression of both chains in substantially equimolar proportions enables optimum yields of functional antibody to be obtained. The two chains assemble within the cell and are then secreted into the culture media as functional antibody. The resulting recombinant antibody may be purified and formulated in accordance with standard procedures.

[0056] One aspect of the present invention includes recombinant non-human primate antibody, more particularly recombinant chimpanzee or old world monkey antibody for example recombinant cynomolgus monkey antibody.

[0057] The invention further comprises a recombinant primate antibody produced or produceable by:

- i) selecting a primate lymphocyte derived cell line that is capable of expressing a desired antibody;
- ii) isolating RNA from the cell-line and separating mRNA from the other RNA so isolated;
- iii) synthesising cDNA from the mRNA and inserting the cDNA into a cloning vector;
- iv) transforming a host cell with the vector containing the cDNA - to obtain a library;
- v) screening the library for cDNA encoding the antibody;
- vi) inserting the cDNA encoding the antibody into an expression vector;
- vii) transfecting a host cell with the expression vector containing the cDNA; and
- viii) culturing the transfected host cell and isolating the desired antibody.

[0058] The use of eukaryotic cell lines transfected with cDNA can be expected to yield greater than 50 μ g/ml of antibody preferably up to or more than 250 μ g/ml.

[0059] A further aspect of the invention comprises a recombinant primate antibody produced or produceable by the process of culturing a eukaryotic host cell line capable of expressing cDNA encoding primate antibody heavy and light chains.

[0060] The resultant antibody can be used as a therapy, according to its specificity. One example provided hereinafter is a human anti-hepatitis A antibody for use in the treatment of hepatitis A infections. Other anti-viral antibodies can

be obtained which target viruses such as other hepatitis viruses (e.g. hepatitis B and C) or herpes viruses : herpes simplex virus, cytomegalovirus, Epstein Barr virus, varicella zoster virus. Anti-HIV antibodies can be obtained according to the invention. These antibodies may be used to treat AIDS, to prevent or delay onset of AIDS in HIV positive or ARC patients, or prophylactically in individuals who have come into contact with the virus through, for example needlestick injury. Another use is in the prevention of transmission of the virus from an HIV positive mother to her infant during pregnancy or at childbirth. This may involve treatment with the antibody before, during and/or after birth.

[0061] Antibodies may also be obtained which target other pathogenic organisms such as bacteria, protozoa etc. Cancerous cells are also possible targets for human antibodies. Optionally the antibodies can be used as targeting moieties which deliver chemical or biological compounds. These are incorporated into the cell by endocytosis where they are toxic or are metabolised to form a toxic agent, killing the cell. Antibodies of the invention may target other anti-self antigens such as those present in autoimmune diseases for example multiple sclerosis or in for example inflammatory disorders such as arthritis. Anti-Rhesus D antibodies cannot be made in animal models - a human antibody would therefore have great value as a diagnostic tool in blood typing and/or could be employed therapeutically. Anti-rhesus D antibody can be administered to a rhesus negative mother at any time during pregnancy or birth to prevent her from raising antibody against the foetus or during subsequent pregnancies. A further aspect of the invention therefore includes the use of a recombinant human antibody in the treatment or prophylaxis of exposure of a rhesus negative individual to rhesus D antigen.

[0062] Antibodies rescued in accordance with the invention could be employed in general diagnostic methods.

[0063] It will be clear from the disclosure that although the invention is primarily concerned with rescue of entire antibody heavy and light chain genes, that fragments such as F(ab) F(ab)₂ and FV can be rescued, expressed and used separately. Such fragments are included within the definition of 'antibody'.

[0064] The present invention therefore provides the use of primate antibodies in the manufacture of a medicament for the treatment of the aforementioned diseases and conditions. Thus, the invention extends to methods of prophylaxis and/or treatment of a human disease and/or condition as described above, comprising administration to a human of an efficacious amount of a primate antibody. Such methods include, methods of diagnosis.

[0065] The dosages of the antibodies will vary with the condition being treated and the recipient of the treatment, but will be in the range 1 to about 100 mg for an adult patient, preferably 1 - 10 mg, usually administered daily for a period between 1 and 30 days. A two part dosing regime may be preferable wherein 1 - 5 mg are administered for 5 - 10 days followed by 6 - 15mg for a further 5 - 10 days.

[0066] Also included within the invention are pharmaceutical formulations containing a recombinant primate antibody. Such formulations preferably include, in addition to antibody, a physiologically acceptable diluent or carrier possibly in admixture with other agents such as other antibodies and/or an antibiotic. Suitable carriers include but are not limited to physiological saline, phosphate buffered saline, glucose and buffered saline. Alternatively, the antibody may be lyophilised (freeze-dried) and reconstituted for use when needed, by the addition of an aqueous buffered solution as described above. Routes of administration are routinely parenteral including intravenous, intramuscular, subcutaneous, and intraperitoneal infection or delivery.

DESCRIPTION OF FIGURES

[0067] Figure 1. Karyotypic analysis of HTOI cells showing polyploid modal numbers of mouse chromosomes and many human chromosomes.

[0068] Figure 2 and Figure 3. Nucleotide and deduced amino acid sequences of Antibody D heavy chain and light chain respectively. The complete sequence of the pH210H2 insert is shown. The signal peptide and CDR sequences are underlined, and the predicted polyadenylation signal overlined. Amino acids are numbered according to Kabat *et al.* (1987).

[0069] Figure 4. Nucleotide alignment of cynomolgus kappa light chain variable regions with human, rabbit and mouse sequences. CDRs are indicated and dots indicate identity to the human Walker sequence. Codons are numbered according to Kabat *et al.*

[0070] Figure 5. Amino acid alignment of cynomolgus kappa light chain variable regions with human, rabbit and mouse sequences. CDRs are indicated and dots indicate identity to the human Walker sequence. Amino acid residues are numbered according to Kabat *et al.*

[0071] Figure 6. Nucleotide alignment of cynomolgus kappa light chain constant region with human, rabbit and mouse sequences. Dots indicate identity to the human germline sequence. Codons are numbered according to Kabat *et al.* One of the ten monkey sequences possessed a G at the nucleotide position underlined.

[0072] Figure 7. Amino acid alignment of cynomolgus kappa light chain constant regions with human, rabbit and mouse sequences. Dots indicate identity to the human germline sequence. Amino acid residues are numbered according to Kabat *et al.*

ExamplesProduction of human/mouse chimaeric cells for hybridisation

5 Example 1: Rescue of human anti-hepatitis A antibody.

a) Production of cell line HTO1

10 [0073] 50 ml blood taken from a healthy human donor, 7 days after booster immunisation with tetanus toxoid, and mixed with preservative-free heparin as anti-coagulant. Mononuclear cells separated on Ficoll/Hypaque (Boyum A. 1986 Scand.J.Clin.Invest. 21,77-89), washed in Hanks buffered saline and fused with a mouse myeloma cell line by conventional techniques as follows:

15 [0074] NS-O mouse myeloma cells (Galfre G. and Milstein C. (1982) Immunology 45, 125-128) were harvested from a log-phase culture and washed in Hanks saline. Mononuclear cells (4.7×10^7) and NS-O cells (6×10^7) were mixed and centrifuged in a 50 ml test tube. The pellet of cells was then resuspended in 1 ml 50% polyethylene glycol solution and mixed gently for 1 minute at room temperature. The fused cells were resuspended in RPMI medium with 10% foetal calf serum and dispensed drop-wise into 60 1 ml aliquots of this growth medium in 24 well plates.

[0075] 24 hours later 1 ml of medium containing hypoxanthine aminopterin and thymidine (HAT) and 1×10^6 Balb/c mouse spleen cells was added to each well. The plate was incubated at 37°C in 5% CO₂.

20 [0076] 20 days after fusion the supernatants were screened by radio-immunoassay for human anti-tetanus toxoid antibodies. One well containing one small colony of cells (approximately 20) was identified. This colony was slow-growing, a characteristic associated with prolonged stability of antibody secretion due to enhanced retention of human chromosomes.

25 [0077] These cells were transferred to a fresh well and after three weeks were cloned by limiting dilution (LD). All the subclones tested were positive in the Radioimmunoassay (RIA).

[0078] One clone, PB47 1.A1.B9.E10, was named HTO1 and cells frozen down. These cells synthesised human IgM anti-tetanus toxoid antibody.

30 [0079] Karyotypic analysis showed that the cells contained a polyploid modal number of mouse chromosomes and many human chromosomes (see Figure 1). These cells were selected as starting material for production of a polyploid fusion partner for the preparation of further hybridomas.

[0080] The cell line HTO1, which secreted human IgM anti-tetanus antibody was used as starting material to produce a polyploid fusion partner. Cells sensitive to HAT were selected by subjecting the aminopterin resistant HTO1 cells to sequential passage through medium containing 8-azaguanine, from 1µg-20µg/ml.

35 [0081] In order to stimulate loss of the human antibody heavy and light genes, a sample of cells was subjected to a 90% lethal dose of ultra-violet irradiation. Irradiated cells were cloned at limiting dilution and a number of colonies were selected on the basis of lack of cytoplasmic staining with anti-human Ig and nuclear size which correlates with chromosome number. One clone, HTO1.A was selected after karyotypic analysis showed it to be polyploid with between 60-140 mouse chromosomes.

40 [0082] Selection of vigorously growing HTO1.A cells was achieved by passaging a sample through the peritoneal cavity of a pristane-primed mouse (PRISTANE is 2,6,10,14 - tetramethylpentadecane from Aldrich). Those cells that survived, grew as single colonies on microtitre plates. These were cultured and assessed for growth, karyotype and fusibility. One, designated HTO1.A.P1 was finally selected on the basis of modal numbers of 135 mouse and 3 human chromosomes. This cell line was used as a fusion partner with the peripheral blood lymphocytes from the hepatitis A virus seropositive donor.

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b) Removal and stabilisation of antibody secreting cells

[0083] A blood sample (30mls) was obtained from a hepatitis A virus (HAV) sero positive donor, approximately four months after an infection with hepatitis A contracted from contaminated food in the UK.

50 [0084] Peripheral blood lymphocytes were separated on a lymphoprep gradient (Flow Labs), transformed with Epstein Barr Virus (EBV) and cultured for ten days in medium containing phaetohaemagglutinin and 10% foetal calf serum. They were then fused with appropriate human/mouse chimeric cells as described above, using PEG 1500, and cultured in the presence of HAT and 10^{-5} M ouabain in 2 ml wells. Supernatant media were screened by sandwich ELISA for anti-HAV activity, ten days post-fusion when distinct colonies were visible microscopically. Individual colonies were picked from positive wells, and monoclonal cell lines established from these by cloning twice from single cells at limiting dilution. Of the original 42 2ml wells, seventeen were strongly positive in initial ELISA screens following extensive re-feeding, but secreting monoclonal lines were only successfully established from four of these, The others ceased secreting antibody at various states of isolation or cloning, including after double cloning, presumably due to the inherent

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chromosomal instability of heterohybrids.

c) Selection of hybridoma

5 ELISA Studies

[0085] The four antibodies (A, B, C & D) from the cell lines described above, were titrated in both Sandwich and Direct ELISA against full and natural empty HAV particles. The titres, expressed as the log 10 reciprocal dilutions producing 50% of the maximum absorbance plateau, are shown in Table 1a. These values, expressed as percentages of the titres of individual antibodies against native particles in the Sandwich test are given in Table 1b.

ELISA TITRES OF HUMAN ANTIBODIES AGAINST FULL AND NATURAL EMPTY HAV PARTICLES

[0086]

Table 1a

Antibody I.D.	Sandwich		Direct	
	Fulls	Empties	Fulls	Empties
Antibody A	4.42	4.42	3.77	3.35
Antibody B	4.85	4.78	4.13	3.80
Antibody C	3.91	3.72	4.02	3.59
Antibody D	4.10	4.15	3.92	3.60

ELISA ACTIVITY OF ANTIBODIES EXPRESSED AS PERCENTAGE OF THE HOMOLOGOUS ANTIBODY TITRE AGAINST FULL PARTICLES IN THE SANDWICH TEST

[0087]

Table 1b

Antibody I.D.	Sandwich		Direct	
	Fulls	Empties	Fulls	Empties
Antibody A	100	100	22	9
Antibody B	100	85	19	9
Antibody C	100	65	126	48
Antibody D	100	112	66	32

d) Competition Studies

[0088] The ability of the antibodies to inhibit both each other and murine antibodies from binding to the virus was carried out using solid phase radioimmunoassay (RIA) and ELISA techniques, which only differ at the final stage. The results, expressed in Table 2 as the maximum competition (%) obtained between antibody pairs, show that;

I) Antibodies A and B are indistinguishable and are similar to the K24F2 murine antibody (MacGregor A. et al 1983, J.Clin.Microb. 18 page 1237).

II) Antibody D is closer in nature to murine antibody B5B3, (Stapleton J.T. and Lemmon S.M. 1987 J. Virol. 61 p491) and only interferes with Antibody A to a maximum of approximately 30% in reciprocal tests.

III) Antibody C appears to be functionally intermediate between Antibody A and D.

IV) Both Antibody A and Antibody D were individually able to inhibit the binding of human HAV polyclonal sera (Lemmon S.M. et al 1983 J.Clinical.Microb. 17 page 834 namely-Foxwell and Chulay) very efficiently.

[0089] The high competition values obtained with Antibodies A and B against B5B3 were obtained with 10-fold con-

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centrated antibody, whereas the tissue culture supernates produced only 20% competition or less. In contrast, the same supernates required substantial dilution to obtain full competition curves against the K24F2 and K34C8 antibodies (MacGregor A. et al 1983, J.Clin.Microb. 18 page 1237) as did the Antibody D supernate against B5B3.

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MAXIMUM COMPETITION (%) OF ANTIBODY BINDING TO THE 18F HAV STRAIN

[0090]

Table 2

4th Ab (Detection)						Human Polyclonal Antiserum (Chulay)
	A	D	K34C8	K24F2	B5B3	
3rd Ab (Competitor)						
Antibody A	100	32	90	100	96 ¹	94
Antibody B	100	33	78	100	>65 ¹	
Antibody C	66	55	46	72	91	
Antibody D	24	100	29	69	99	92
Antibody A&D 1:1 mix						99
K34C8	100	32	100			
K24F2	100	70		100		
B5B3	69	100			100	
Human polyclonal antiserum (NF)						100

(1) Concentrated antibody required for maximum competition

e) RIFA STUDIES

[0091] All antibodies detected by the initial screening ELISA were also positive in Radioimmunofocussing Assay against the 18f virus. (Daemer R.J. *et al* (1981). *Infect & Immunol.* 32 page 388; and Stapleton J.T. and Lemmon S.M. (1987) *J.Virol* Vol 61 p492; and Ping L.A. *et al* 85 p821). The reductions in virus RIFA titres, obtained from reacting equal volumes of a fixed concentration (1mg/ml) of affinity purified antibody with 10-fold dilutions of the 18f and 43c virus strains (43c strain was derived from 18f strain by passaging under pressure from a murine monoclonal), are summarised in Table 3. These demonstrate that mutant 43c is very poorly neutralized by either antibody but shows significant, albeit reduced neutralization with polyclonal Foxwell serum.

[0092] Although both antibodies appear to 'neutralize' 18f virus far less efficiently than polyclonal serum, this is largely due to a fairly constant number of residual plaques surviving at each virus dilution and consequently distorting the Spearman-Kärber calculation of titre. Reaction with polyclonal serum, however, eliminated all plaque formation. Similar reaction slopes of the antibodies to polyclonal sera in competition studies, and their overall ELISA reactivity, give no indication that these represent low affinity antibodies.

REDUCTION IN TITRE LOG₁₀ PLAQUE FORMING UNITS/ML OF HAV ISOLATES REACTED WITH SPECIFIC ANTIBODY

[0093]

Table 3

	A	Antibody D	Polyclonal
HAV strain	(1mg/ml)	(1mg/ml)	human serum, (1/10)
18f	3.15	2.72	>4.56
43c	0.68	0.78	1.93

f) Cloning and sequencing of the anti-hepatitis A virus monoclonal Antibody D heavy and light chains

Method 1

[0094] Total RNA was isolated from 2.5×10^7 Antibody D expressing cells following the method (1) of Chomczynski and Sacchi (1987 *Anal. Biochem.* 162, 156-159), using 1ml of extraction solution per 1×10^7 cells. The resulting RNA pellet was redissolved in 50µl diethyl pyrocarbonate (DEPC)-treated distilled water, and spectrophotometrically determined to be at a concentration of 4.4µg/µl. Dynabeads Oligo (dT)₂₅ (Dynal Winal, UK) was used to extract polyadenylated RNA from 75µg total RNA employing the manufacturer's protocol.

Method 2

[0095] An alternative method (2) for isolation of polyadenylated RNA involved isolation directly from 10^3 Antibody D secreting cells using the Micro-FastTrack mRNA isolation system (Invitrogen, San Diego, USA) following the method recommended by the manufacturer.

[0096] The ability to clone human antibody genes from a limited number of cells was investigated. A cDNA library was generated with polyadenylated RNA isolated from one thousand secreting antibody D cells using Method 2 above. The library was calculated to possess a potential of about 2000 size-selected (>500bp) cDNA inserts. Half of the library was plated and screened for human κ L chain, and 2 positive clones detected. Partial sequencing confirmed that both clones possessed full length L chain genes, incorporating signal peptide sequences. Although this library was not screened with the H chain probe, full length H chain inserts should be present as calculated from the frequency of H chain positives (2/500) obtained from the initial cDNA library.

[0097] cDNA was synthesised from the isolated mRNA and cloned into the plasmid pSPORT-1 using the SUPERSCRIPT Plasmid System for cDNA Synthesis and Plasmid Cloning kit (GIBCO/BRL, Paisley, UK) following the method recommended by the manufacturer. *Escherichia coli* MAX EFFICIENCY DH5 α Competent Cells (GIBCO/BRL) were transformed with the resulting cDNA/pSPORT-1 ligation. Approximately 4500 colonies were lifted onto Hybond-N nylon filters (Amersham) and lysed, denatured and fixed following the method of Buluwela *et al.* (*Nucleic Acids Res*, 1989 17 452). The filters were treated with proteinase K (50µg/ml in 0.2xSSC, 0.1% SDS at 55°C for 30 min), and then excess debris removed with a tissue. The libraries were then screened for human antibody heavy, and light chain sequences using heavy and light chain probes.

[0098] The heavy chain probe was a human IgG1 antibody cDNA insert (rat CAMPATH-1 [Campath is a trademark of The Wellcome Foundation Limited] antibody heavy chain CDRs reshaped on human NEW IgG1 antibody heavy chain; Riechmann *et al.*, 1988 Nature 382 323-327) which was labelled with digoxigenin-11-dUTP using the Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim) and employed to screen filters, possessing approximately 500 lifted colonies, for Antibody D heavy chain following the manufacturer's protocol. Two potential positive colonies were detected and selected for further analysis. Plasmid DNA was prepared using the QIAGEN PLASMID KIT (DIAGEN, DUSSELDORF, FRG) or the method of Del Sal *et al.* (1988 Nucleic Acids Res 16 9878) and both of the potential positive clones were found to contain inserts of the expected size for human immunoglobulin heavy chain cDNA. A clone, pH210H2, was selected, and sequenced in both directions by plasmid priming following the dideoxy chain termination method (Sanger *et al.*, Proc. Natl. Acad Sci USA, 1977 74 5463-5467), according to the Sequenase kit (United States Biochemicals - USB Cleveland, USA) protocol. The C region of the heavy chain was determined to be $\gamma 1$. The sequence of the variable region is shown in Figure 2.

[0099] The light chain probes were human lambda cDNA (humanised anti-CD3 mAb λ L chain insert [rat anti-CD3 mAb L chain CDR's reshaped on human A Kern'02' Ab L chain: E Routledge Eur.J.Immunol. 1991; 21:2717) and (a Campath-1H κ L chain cDNA insert (rat Campath 1 mAb L chain CDR's reshaped on human RE1 κ AB L chain; Page and Sydenham Bio/technology 1991; 9:64;) which was labelled with digoxigenin-11-dUTP using the Nonradioactive DNA Labelling and Detection Kit (Boehringer Mannheim, Lewes, UK) and employed to screen filters, possessing approximately 4000 lifted colonies, for antibody D light chain following the manufacturer's protocol. Twenty potential positive colonies were detected and 10 selected for further analysis. Plasmid DNA was prepared using the QIAGEN Plasmid kit (DIAGEN, Dusseldorf, FRG) or the method of Del Sal *et al.* (1988) and 8 contained inserts of the expected size for human antibody light chain cDNA. A clone, pH210L2, was selected, and sequenced in both directions by plasmid priming following the dideoxy chain termination method (Sanger *et al.*, 1977), according to the Sequenase kit (USB, Cleveland, USA) protocol. The light chain was determined to be a λ sequence the variable region of which is shown in Figure 3.

g) ASSEMBLY OF EXPRESSION CONSTRUCTS

Example g.i)

[0100] The expression vector pRDNI was adapted from the pLD9 plasmid (described in Page, M. and Sydenham M. A. (1991) Biotechnology 9 64-68) as follows. The HindIII site used to insert the SV₄₀ origin of replication and the other HindIII site 5' to the DHFR coding sequence were destroyed by HindIII digestion, filled-in with klenow fragment of DNA polymerase, and re-ligated. A clone lacking both restriction sites was digested with EcoRI, filled-in with klenow enzyme and re-ligated. The resulting plasmid, lacking all internal HindIII and EcoRI sites, was used to insert the human β Actin expression cassette downstream of the DHFR transcript unit. This plasmid, has a functional SV40 origin of replication and pRDNI has unique HindIII and EcoRI restriction sites downstream of the β Actin promoter. The adapted pRDNI vector was digested with EcoRI, blunted with Klenow enzyme and dephosphorylated using calf intestinal phosphatase. The Antibody D heavy and light chain inserts were cut out of their respective clones, pH210H2 and pH210L2, using HindIII and EcoRI, and blunt ended with Klenow enzyme. The blunt ended inserts were ligated into pRDNI and used to transform Escherichia coli MAX Efficiency DH5 Competent Cells (Bethesda Research Labs BRL). Small-scale plasmid preparations (Del Sal, G. *et al.* (1988) Nucleic Acids Res. 16 9878) were carried out on a number of the resulting colonies and the inserts orientated using appropriate restriction digests. Plasmid DNA was prepared from one heavy and one light chain clone (pRDHH9 and pRDHL27 respectively) using QIAGEN (trademark) columns (Hybaid) following the manufacturer's protocol.

Transfection of COS cells

[0101] 2×10^5 COS cells were plated in D-MEM (plus serum) in each well of a 12-well tissue culture dish. After 24 hours, the cell monolayers were rinsed twice with serum-free medium followed by 0.5 ml of serum-free medium containing 1 μ g of each DNA construct (pRDHH9 and pRDHL27) and 5 μ g TRANSFECTAM (Northumbria Biologicals Limited), as recommended by the manufacturer. After further incubation for 6 hours, the transfection medium was aspirated and replaced with 1 ml D-MEM (plus serum). After 48-72 hours, the medium was removed and assayed for human antibody.

ELISA Assay for Human Antibody

[0102] The medium from the COS cell transfection was assayed for the presence of, human antibody. In the absence of light chain synthesis, heavy chains are not secreted from a cell and are degraded internally (Hendershot L. *et al.*

Immunol. Today 8 111-114). Antibody can thus be assayed by detection of heavy chain in the culture medium. Microtitre plates were coated with anti-human IgG and incubated with the culture medium. Antibody was detected by visualisation with an anti-human gamma chain specific peroxidase conjugate.

5 Example g.ii)

[0103] The DNA encoding the H and L chains was cloned into the pEE6hCMV (Stephens and Cockett Nucl. Acids Res. M : 7110 1989; Bebbington et al. Biotechnology 10 169 1992) and pEE12 (Rolfe unpublished) expression vectors respectively. Plasmid pEE12 contains a cDNA encoding the hamster glutamine synthetase gene (GS; a marker that
10 can be selected and amplified using the toxic glutamate analogue, L-methionine sulfoximine [MSX]) under the control of the SV40 early promoter and SV40 splicing and polyadenylation signals obtained from pSv2.GS (Bebbington and Hentschel DNA cloning volume III New York Academic Glover DM ed. 1987). Downstream of this selection cassette is the complete enhancer, promoter and 5' UTR from the major immediate early (MIE) gene of the human cytomegalovirus (hCMV), and this is used to drive expression of the antibody gene. This expression cassette with its associated
15 origin of replication and β -lactamase gene were obtained from pEE6.HCMV (Stephens and Cockett Nucl. Acids Res. 17 : 7110 1989). Cells transfected with vectors pEE6hCMV and pEE12 are therefore capable of growth in glutamine minus medium. Plasmids pEE6hCMV and pEE12 were obtained from Celltech Ltd, Slough, Berkshire, SL1 4EN, UK).
[0104] Recombinant plasmids (5 μ g of each) were transfected into 5×10^5 COS-1 cells or 10^7 YO myeloma cells using the Transfectam reagent (Promega, Southampton, UK) under the conditions recommended by the manufacturer.
20 As a result, the selection is only on the pEE12 plasmid and effective expression relies upon co-integration of the two plasmids.

[0105] H and L chains were co-transfected in COS cells to ensure that the constructs were correctly inserted in frame, efficiently transcribed and translated, and that the resulting human antibody was properly secreted. Having confirmed transient expression of the antibody by ELISA the plasmids were co-transfected into YO myeloma cells.

25 [0106] Stock COS-1 cells (Source ECACC, Porton Down, UK) were maintained in DMEM medium (Flow, Irvine, UK) supplemented with 10% foetal calf serum (APP, Dudley, UK). COS cell transfections were carried out in DMEM medium (Flow, Irvine, UK).

[0107] Stock YO cells (Source ECACC, Porton Down, UK) were maintained in complete medium containing DMEM medium (Flow, Irvine, UK; without glutamine and ferric nitrate but with sodium pyruvate [110mg/L]; GIBCO/BRL, Paisley, UK) 1 x non-essential amino acids (Flow, Irvine, UK) and 10% foetal calf serum (APP, Dudley, UK). Transfected cells
30 were transferred to 96 well plates at densities of 3×10^5 , 7.5×10^4 and 1.5×10^4 cells/ml in 50 μ l complete medium and incubated at 37°C for 24 hours. Subsequently, 100 μ l of select medium containing DMEM medium (Flow, Irvine, UK; without glutamine and ferric nitrate but with sodium pyruvate [110mg/L]; GIBCO/BRL, Paisley, UK) supplemented with glutamate (60 μ g/ml), asparagine (60 μ g/ml; Sigma, Poole, UK), 1 x non-essential amino acids, 7mg/L of adenosine,
35 cytidine, guanosine and uridine, 2.4mg/L of thymidine (Sigma, Poole, UK), 10% dialyzed foetal calf serum (APP, Dudley, UK) and 4 μ M MSX (to titrate out the endogenous glutamine synthetase enzyme of YO cells) was added in order to select clones which had integrated the transfected plasmids containing the human antibody genes.

[0108] Growth media from COS-1 cells four days post transfection and from YO cells grown in medium for selection of plasmid integration was assayed by a sandwich ELISA assay using flexible microtitre plates (Falcon, Becton-Dickinson, Plymouth, UK) coated with polyclonal anti-human IgG (Sigma, Poole, UK) as capture antibody. The assay sample
40 was added and detection performed with an anti-human γ chain specific peroxidase conjugate (Sigma, Poole, UK) and orthophenylene diamine-HCl (Sigma, Poole, UK) as substrate. Positive clones were transferred to 24 well plates for further propagation in select medium.

45 [0109] Three clones capable of growth in levels of MSX toxic to untransfected YO cells were obtained. Two of these were secreting human antibody, as determined by ELISA assay, whilst the other line appeared to be a false positive.

EXAMPLE 2 : Determination of cynomolgus monkey antibody chain sequences

a) Ouchterlony Immunodiffusion

50 [0110] An Ouchterlony immunodiffusion test using serum from chimpanzee cynomolgus and aotus monkeys was performed to give an indication of the similarity which might exist between protein sequences of human and primate immunoglobulins.

[0111] Micro-ouchterlony plates - supplied by The Binding Site.

55 Sheep anti-human IgG1, IgG2, IgG3 and IgG4 - The Binding Site.

Goat anti-human IgM and Goat anti-human IgA - supplied by Sigma.

Sheep anti-human Ig κ light chain - Serotec.

Sheep anti-human Ig λ light chain - Serotec.

[0112] Sera from cynomolgus (old world) monkey, aotus (new world) monkey and chimpanzee (ape) were tested for reaction against a series of anti-human immunoglobulins. The undiluted sheep anti-human antibody (10 μ l) was placed in the centre well of an ouchterlony plate and the primate sera (10 μ l) placed in the surrounding outer wells. All sera were diluted in PBS according to the manufacturers recommendations. Rabbit and mouse sera were included in the assay as was human serum which acted as a positive control. The plate was incubated at room temperature under humid conditions overnight or until precipitin bands could be visualised.

[0113] Precipitin bands were formed between chimpanzee serum and all anti-human sub-classes. Some but not all human Ig classes, namely IgG1, IgM, IgA and κ and λ light chains formed precipitin bands with cynomolgus monkey serum. Aotus monkey serum (a new world monkey) was recognised by the least human antibodies - the only strong reaction was seen with the κ and λ light chains. Surprisingly, the rabbit serum reacted with the anti-human λ light chain - this suggests that there may be a shared epitope between rabbit and human Ig protein sequences for recognition to occur. Mouse serum did not recognise any human Ig classes at all.

[0114] This test gave the expected results considering the evolutionary relationship of each species to man - ie. the new world monkeys diverged earlier than the old world monkeys, which show greater protein homology to human. To investigate the degree of nucleotide homology between primate and human Ig genes, the κ light genes from the old world, cynomolgus, monkey were chosen for this study.

b) Cynomolgus monkey total RNA was prepared from 10 mls of peripheral blood lymphocytes using the guanidium thiocyanate method of extraction REF.

[0115] First strand cDNA was synthesised from total RNA using the BRL SUPERScript system (BRL). Total RNA (5 μ g) in a volume of 13 μ l was added to 1 μ l of oligo-dT primer and allowed to anneal by heating at 70°C for 10 minutes followed by cooling on ice.

[0116] First strand cDNA was reverse transcribed by addition of 2 μ l reaction buffer, 1 μ l dNTPS, 2 μ l DTT and 1 μ l Reverse transcriptase. The reaction was incubated at room temperature for 10 minutes, followed by 50 minutes at 42°C. The reaction was stopped by heating at 90°C for 5 minutes, then placing on ice for 10 minutes. The mRNA template was digested with 1 μ l of RNase H for 20 minutes at 37°C.

c) PCR AMPLIFICATION OF FIRST STRAND cDNA

PCR primers

[0117] 151 - homologous to the 5' end(FR1) of human V κ l

5' GACATTCAGCTGACCCAGTCTCCA SEQ ID NO:1

[0118] 301 - homologous to the 3' end of human C κ (contains HindIII restriction site)

5' GATCAAGCTTCTAACACTCTCCCC SEQ ID NO:2

[0119] These primers and all subsequent PCR and sequencing primers mentioned were synthesised on an oligodeoxynucleotide synthesiser and were used at a concentration of 200 ng/ μ l.

[0120] First strand cDNA was directly amplified by PCR, without the need for second strand synthesis, using primers 151 and 301. The former is specific for the 5' end of the variable region(FR1) of the human κ 1 light chain, and the latter is specific for the 3' end of the constant region of human κ light chain.

[0121] The light chain was amplified in the following reaction; the complete first strand cDNA reaction mixture was added to 21 μ l dH₂O, 8 μ l synthesis buffer [(Boehringer)], 4 μ l primer 301, 4 μ l primer 151 and 0.5 μ l Taq polymerase. This mixture was overlaid with mineral oil and subjected to 20 cycles of PCR using the previously mentioned program. The reaction was checked on a 1% agarose gel as before.

d) CLONING OF CYNOMOLGUS κ LIGHT CHAINS

[0122] PCR primer 260 - homologous to the 5' end of human V κ (contains Hind III restriction site).

5' GATCAAGCTTGACATTGACCTGACCCAGTCTCCA SEQ ID NO:3

i. Introduction of Hind III sites at either end of the light chain fragment

[0123] The cynomolgus monkey κ light chain cDNA obtained from the previous PCR was cloned in to the HindIII site of pUC18 Maniatis T. et al J.Molecular Cloning (Cold Spring Harbour Laboratory Press 1989). The amplified light chain contains a HindIII site at the 3' end of the constant region due to the sequence of primer 301, but lacks such a restriction site at the 5' end. Therefore, to enable the light chain to be cloned directly into pUC18, a HindIII site was introduced at the 5' end of the variable region by means of a second PCR using primers 260 and 301. The former has an identical sequence to primer 151, but with a HindIII site added on to the 5' end.

[0124] The reaction was set up as previously described using 1 μ l of cynomolgus cDNA (from PCR mix) in a final volume of 100 μ l. The DNA was amplified by 20 cycles of PCR, then checked on a 1% agarose gel. The gel showed many other bands other than those expected for the amplified light chain. It is possible that some of the smaller bands could be due to the primers annealing to each other, forming so-called primer-dimers.

ii) Purification of the PCR product

[0125] The light chain was purified from the PCR mixture before cloning in to pUC18, in the following way; the PCR reaction was frozen at -20°C, and the liquid mineral oil aspirated off. The DNA was then extracted twice with phenol/chloroform, followed by a single extraction with chloroform alone. The solution was adjusted to 5mM EDTA, 10mM Tris pH8, 0.5% SDS and proteinase K added to 50 μ g/ml. The reaction was incubated at 37°C for 30 minutes, then 68°C for 10 minutes. A second phenol/chloroform extraction. The DNA was precipitated by the addition of 1/10 volume 3M sodium acetate and 2.5 volumes of absolute ethanol (1 μ l dextran sulphate was added as a carrier). The DNA was placed at -20°C for 30 minutes, then pelleted in an eppendorf centrifuge. The pellet was washed in 70% ethanol, dried and resuspended in 17 μ l water. This purification process overcomes the problem of Taq polymerase remaining bound to the DNA and thus inhibiting restriction enzyme activity.

iii) Preparation of the HindIII fragment for cloning

[0126] The light chain DNA was then digested with restriction enzyme HindIII, in order to clone in to the HindIII site of pUC18. To 17 μ l of light chain DNA, 2 μ l of buffer B and 1 μ l of high concentration HindIII was added and incubated at 37°C for one hour. The digest was separated on a 1% agarose gel and the band corresponding to the light chain was excised from the gel. The DNA was purified from the agarose using "Prep-a-gene" purification kit (Biorad Ltd) following the manufacturers instructions.

e) LIGATION OF CYNOMOLGUS LIGHT CHAIN IN TO PUC18

[0127] The purified HindIII fragment was cloned in to the HindIII site of pUC18 using the following method; 20 μ l of cynomolgus DNA (from a volume of 80 μ l) was added to 1 μ l of HindIII-digested pUC18 (50 μ g/ μ l Pharmacia), 3 μ l ligase buffer (Boehringer), 3 μ l T4 DNA ligase (Boehringer) and 3 μ l distilled water. The reaction was incubated at 15°C for 3 hours.

f) TRANSFORMATION OF DH5COMPETENT CELLS BY pUC18- κ CHAIN

[0128] E.coli DH5 α maximum efficiency cells (BRL) were transformed with the light chain plasmid. 100 μ l of cells were thawed slowly from -70°C, mixed gently with 5 μ l ligation reaction and kept on ice for 30 minutes. The cells were incubated at 42°C for 45 seconds then cooled on ice for 2 minutes. 1ml of SOC medium (20g bactotryptone, 5g yeast extract, 0.5g NaCl, 10ml 250mM KCl, 5ml 2M MgCl, 20mM glucose per litre) was added and incubated at 37°C for 1 hour. The transformation reaction was plated out on to five LB plates (LB agar plates: 12g tryptone, 24g yeast extract, 4ml glycerol, 100ml phosphate buffer, 15g bacto-agar per litre) containing 100 μ g/ml ampicillin and incubated overnight at 37°C. The number of colonies were counted per plate and the transformation efficiency calculated.

[0129] Five colonies were picked from the plates and the presence of light chain inserts checked by PCR. Each colony was resuspended in 65.5 μ l distilled water and subjected to 20 cycles of PCR using primers 260 and 301 in the standard reaction mixture. The PCR reactions were run on gel as before. Four out of five colonies were found to contain the insert which indicated that the cloning had been successful.

g) PLASMID MINIPREPS OF PUC18-κCHAIN

[0130] Twenty colonies from the five transformation plates were picked and inoculated in to 2ml volumes of L-Broth (L broth - as LB plates but without agar) containing 100µg/ml ampicillin. The cultures were incubated at 37°C overnight with shaking. 1.5ml of each culture was spun down and the cell pellet resuspended in 200µl STET - 0.1M NaCl, 10mM Tris HCl pH8, 1mM EDTA, 5% Triton-X-100) with 1mg/ml lysozyme. The tubes were incubated for 5 minutes at room temperature then boiled for 45 seconds and centrifuged for 10 minutes. The resulting pellet was removed with a sterile toothpick and 8µl of 8% CTAB added to the supernatant. The tube was spun for 5 minutes to pellet the plasmid.

[0131] DNA which was subsequently resuspended in 300µl of 1.2M sodium chloride by vortexing. The DNA was precipitated by the addition of 750µl ethanol, vacuum dried and resuspended in 20µl of (TE buffer TE - 10mM Tris pH7.4, 1mM EDTA).

[0132] The minipreps were checked for the presence of the light chain by restriction enzyme digestion (HINDIII restriction enzyme and Buffer B-10u/µl - Boehringer). 2µl of each DNA was added to 0.5µl of buffer B, 1µl of distilled water, 1µl of RNase A (500µg/ml Boehringer) and 0.5 µl HindIII. The digests were incubated at 37°C for 1.5 hours then run on a 1% gel. The presence of the light chain was indicated by a fragment of approximately 700 base pairs.

h) SEQUENCING OF THE κ LIGHT CHAINSSequencing primers

[0133]

M13 Reverse primer - anneals to -ve strand of pUC18, downstream of the polycloning site - supplied by Pharmacia;

5' AACAGCTATGACCATG SEQ ID NO:4

-40 forward primer - anneals to +ve strand of pUC18, downstream of the polycloning site - supplied by USB

5' GTTTTCCCAGTGACGAC SEQ ID NO:5

299 - human Cκ region primer

5' GCGTCAGGGTGCTGCTGAGG SEQ ID NO:6

106 - human Cκ region primer (5' end)

5' GGCGGGAAGATGAAGACAGA SEQ ID NO:7

151 and 260 - see stages C & D

261 - human Cκ region primer

5' TTCAGCAGGCACACAACAGA SEQ ID NO:8

i) Ten clones from the previous stage were chosen for sequencing (clones 1,2,4,5,9,12,14,15,18 and 20), by the dideoxy chain termination method. The remaining 18µl of miniprep DNA was added to 2µl NaOH(2M) and heated at 68°C for 20 minutes in order to denature the DNA. The DNA was cooled and precipitated by the addition of 8µl 5M Ammonium

acetate (pH5.4) and 100 μ l ethanol on dry ice for 5 minutes. The DNA was pelleted, washed in 70% ethanol, vacuum dried and resuspended in 20 μ l distilled water.

[0134] An aliquot of DNA (7 μ l) was added to 2 μ l of reaction buffer and 1 μ l of the appropriate primer and incubated at 65°C for 2 minutes, then cooled slowly to below 30°C to anneal primer and template. It was necessary to use several different primers in order to sequence the entire κ light chains, the details of which are set out above. To the template/primer the following was added; 1 μ l DTT (0.1m USB), 2 μ l labeling mix (5 x USB), 0.5 μ l ³⁵S-dATP (10 μ Ci/ μ l Amersham) and 2 μ l Sequenase enzyme. The reaction was incubated at room temperature for 5 minutes in order to synthesise an ³⁵S-labelled leader sequence. When the labelling reaction was complete, 3.5 μ l of labelling mix was added to four wells of a multiwell plate containing 2.5 μ l of ddGTP, ddATP, ddTTP and ddCTP. The chain termination reaction was allowed to proceed for 5 minutes at 37°C before 4 μ l of stop solution was added to each well. The reactions were stored on ice until required.

[0135] An 8% acylamide gel (ultra-pure acylamide gel mix-8 supplied by BRL) was poured between siliconised glass plates and pre-run at 40mA for one hour using TBE (0.09M TMS-borate pH8, 0.002M EDTA) as a run buffer. The wells were washed out with buffer before and after the pre-run to remove air bubbles and urea. Before loading the gel, the samples were heated at 95°C for 2 minutes, then 4 μ l of each sample was loaded on to the gel. The samples were run at 40 mA for 1.5 hours, then a second loading applied to the gel and run for another 1.5 hours. This gave a short and long run for each set of samples. The plates were disassembled and the gel placed in 10% acetic acid/10% methanol before transferring the gel to a sheet of 3MM paper. The gel was dried at 80°C for 1.5 hours and exposed to X-ray film overnight at room temperature. The autoradiographs were developed and the sequence read from the bottom upwards, with the long and short runs overlapping.

j) COMPARISON OF SEQUENCE DATA

[0136] The complete light chain sequences of the ten clones were obtained by running a series of sequencing reactions using different primers. Comparisons of sequence data are shown in Figures 4-7. Clones 14 and 5 were found to contain truncated light chain sequences. This is most likely to be an artifact caused by the reverse transcription of mRNA or the PCR amplification of the cDNA. It may be possible that the mRNA or cDNA formed a secondary structure through which the appropriate enzyme could not read, thus resulting in a partial sequence. Clone 15 was chosen for further comparison with known light chain sequences to confirm its identity and human, rabbit and mouse light chains to determine nucleotide and amino acid homology, the results of which can be seen in Tables 4-7.

[0137] The cynomolgus light chain constant region was compared to a human germline C κ gene (Hieter, P.A. *et al* Cell **22**, 197 (1980)), a mouse MOPC21 C κ mRNA (Hamlyn, P.A. *et al* Nucl. Acids Res. **9** 4485 (1981) and a rabbit C κ mRNA (17D9) (McCartney - Francis N. *et al* Proc. Natl. Acad. Sci. **81** 1794, 1984). The cynomolgus variable regions were compared to a human lymphoid cell line, Walker (Klobek, H.G. *et al* Nucl. Acids Res. **12** 6995, 1984). This cell line expresses a light chain gene which belongs to the V κ 1 family and which utilises the J5 gene segment. The 17D9 rabbit sequence was used to compare cynomolgus variable sequences as was the S107A mouse V κ 1 sequence (Kwan, S.P. *et al* J.Exp. Med. **153**, 1366 (1981)). This is a phosphocholine binding IgA κ myeloma which utilises the mouse J1 gene segment. The variable region of clone 15 was also compared to a human antibody, Daudi (Klobek 1984). This light chain gene belongs to the V κ 1 family and uses the J5 gene segment. The cynomolgus antibody κ light chain constant region sequence shows high homology to the human C κ gene at both the nucleotide (91.6%) and the amino acid (81.3%) levels. Mouse C κ appeared to be more homologous to either cynomolgus monkey or human C κ than did rabbit. However, percentage homologies did not appear to differ dramatically when comparing human and monkey to rabbit and mouse or indeed rabbit to mouse.

[0138] Tables 5 and 6 show percentage homologies for each framework region and each CDR of clone 15. As expected, the framework regions are relatively conserved between species compared to CDRs where there is considerable variability. For example, clone 15 shares 90-96% amino acid sequence homology with human in frameworks 1 and 3 but only 22% homology within CDR 3. Clone 15 shows a high degree of homology to both Walker and Daudi human light chain sequences especially within frameworks 1 and 3, although exhibits a higher homology to Walker than Daudi. The rabbit V κ sequence shows more homology to both human and monkey sequences than does mouse. This could explain why some reaction to anti-human antibodies was seen with rabbit serum in the ouchterlony test but no reaction was seen with mouse serum.

Table 4

Comparisons of light chain constant region sequence homologies		
Species	% homology DNA	% homology AA
Cynomolgus / human	91.6	81.3
Cynomolgus / rabbit	66.0	51.0
Cynomolgus / mouse	68.0	58.0
Human / rabbit	65.7	45.8
Human / mouse	69.8	58.9
Rabbit / mouse	63.0	49.5

Table 5

Percentage homologies of light chain framework and CDR DNA sequences				
	Cyno 15 v			
	Human W	Human D	Rabbit	Mouse
FR 1	89.9	89.9	71.0	66.7
CDR1	84.8	66.7	60.6	42.4
FR 2	95.6	80.0	86.7	75.6
CDR2	80.0	60.0	60.0	50.0
FR 3	92.6	91.6	82.1	76.8
CDR3	50.0	61.5	26.9	57.7
FR 4	75.8	75.8	66.7	66.7

Table 6

Percentage homologies of light chain framework and CDR amino acid sequences				
	Cyno 15 v			
	Human W	Human D	Rabbit	Mouse
FR 1	95.7	95.7	52.2	47.8
CDR1	72.7	27.3	36.4	36.4
FR 2	73.3	86.7	66.7	60.0
CDR2	71.4	42.9	71.4	28.6
FR 3	90.6	96.9	78.1	78.1
CDR3	22.2	22.2	0.0	33.3
FR 4	80.0	70.0	60.0	70.0

Claims

1. A process for the production of a recombinant primate antibody comprising:

- (i) selecting a primate lymphocyte-derived cell line that is capable of expressing a desired antibody;
- (ii) isolating RNA from the cell line and separating mRNA from the other RNAs so isolated;
- (iii) synthesising cDNA from the mRNA and inserting the cDNA into a cloning vector;
- (iv) transforming a host cell with the vector containing the cDNA to obtain a library;
- (v) screening the library for cDNA encoding the entire constant and variable regions of the antibody heavy

and light chain genes;

(vi) inserting the cDNA encoding the entire constant and variable regions of the antibody heavy and light chains into an expression vector;

(vii) transfecting a host cell with the expression vector containing the cDNA; and

(viii) culturing the transfected host cell and isolating the desired antibody.

2. A process as claimed in Claim 1 wherein the primate is a human.

3. A process as claimed in Claim 1 wherein the primate is a chimpanzee or an old world monkey.

4. A process as claimed in any of Claims 1-3 wherein the cell-line is produced from lymphocytes from an individual known to have recovered or be in remission from a disease state.

5. A process as claimed in any of Claims 1-3 wherein the cell-line is produced from lymphocytes from an individual known to be infected with a pathogenic organism or is suffering from cancer or an autoimmune disease, but who does not manifest full disease symptoms.

6. A process as claimed in Claims 4 or 5 wherein the individual has been infected by a virus.

7. A process as claimed in any of Claims 1-3 wherein the cell-line is produced from lymphocytes from an individual who has been vaccinated or inoculated with antigen and has mounted an antibody response.

8. A process as claimed in any of the preceding claims wherein the cell-line is stabilised or immortalised.

9. A process as claimed in any of Claims 1-8 wherein the cell-line is selected by screening for production of antibody with affinity for a desired antigen.

10. A process as claimed in Claim 9 wherein the cell-line is further selected by screening for antibody functionality.

11. A process for the production of a recombinant antibody comprising:

i) micro-RNA preparation from approximately 1000 hybridoma cells;

ii) generation of a size-selected cDNA library;

iii) screening the library for cDNA encoding the entire constant and variable regions of the antibody heavy and light chains and isolating the same;

iv) inserting the cDNA encoding the entire constant and variable regions of the antibody heavy and light chains into an expression vector;

v) transfecting a host cell with the expression vector containing the cDNA; and

vi) culturing the transfected host cell and isolating the desired antibody.

12. A vector suitable for transfecting of a host cell comprising cDNA encoding the entire constant and variable regions of the primate antibody heavy and light chains.

13. A eukaryotic cell-line transfected with cDNA for the expression of the entire constant and variable regions of the primate antibody heavy and light chains.

14. A process for the expression of cDNA encoding the entire constant and variable regions of the primate antibody heavy and light chains, comprising transfecting a eukaryotic host cell with a vector or vectors suitable for the expression of said cDNA.

Patentansprüche

1. Verfahren zur Herstellung eines rekombinanten Primaten-Antikörpers, umfassend:

- (i) Auswählen einer aus Lymphozyten eines Primaten stammenden Zelllinie, die zur Expression eines gewünschten Antikörpers fähig ist;
- (ii) Isolieren der RNA aus der Zelllinie und Abtrennen der mRNA von den anderen so isolierten RNAs;
- (iii) Synthetisieren von cDNA aus der mRNA und Inserieren der cDNA in einen Klonierungsvektor;
- (iv) Transformieren einer Wirtszelle mit dem Vektor, der die cDNA enthält, um eine Bank zu erhalten;
- (v) Durchmustern der Bank auf cDNA, die die gesamten konstanten und variablen Regionen der Gene für die schwere und leichte Kette des Antikörpers codiert;
- (vi) Inserieren der cDNA, die die gesamten konstanten und variablen Regionen der schweren und leichten Ketten des Antikörpers codiert, in einen Expressionsvektor;
- (vii) Transfizieren einer Wirtszelle mit dem Expressionsvektor, der die cDNA enthält; und
- (viii) Kultivieren der transfizierten Wirtszelle und Isolieren des gewünschten Antikörpers.

2. Verfahren gemäss Anspruch 1, worin der Primat ein Mensch ist.

3. Verfahren gemäss Anspruch 1, worin der Primat ein Schimpanse oder ein Altwelt-Affe ist.

4. Verfahren gemäss einem der Ansprüche 1 bis 3, worin die Zelllinie aus Lymphozyten aus einem Individuum hergestellt wird, von dem bekannt ist, dass es sich von einem Krankheitszustand erholt hat oder in der Remission davon befindet.

5. Verfahren gemäss einem der Ansprüche 1 bis 3, worin die Zelllinie aus Lymphozyten aus einem Individuum hergestellt wird, von dem bekannt ist, dass es mit einem pathogenen Organismus infiziert ist oder an Krebs oder einer Autoimmunerkrankung leidet, das aber nicht die vollen Krankheitssymptome offenbart.

6. Verfahren gemäss Ansprüchen 4 oder 5, worin das Individuum mit einem Virus infiziert wurde.

7. Verfahren gemäss einem der Ansprüche 1 bis 3, worin die Zelllinie aus Lymphozyten aus einem Individuum hergestellt wird, das geimpft oder mit Antigen geimpft wurde und eine Antikörperreaktion aufgebaut hat.

8. Verfahren gemäss einem der vorhergehenden Ansprüche, worin die Zelllinie stabilisiert ist oder sich unbegrenzt vermehrt.

9. Verfahren gemäss einem der Ansprüche 1 bis 8, worin die Zelllinie durch Durchmustern auf die Herstellung von Antikörper mit Affinität für ein gewünschtes Antigen ausgewählt wird.

10. Verfahren gemäss Anspruch 9, worin die Zelllinie ferner durch Durchmustern auf Antikörperfunktionalität ausgewählt wird.

11. Verfahren zur Herstellung eines rekombinanten Antikörpers, umfassend:

- (i) Mikro-RNA-Herstellung aus ca. 1.000 Hybridomzellen;
- (ii) Erzeugung einer grössenselektierten cDNA-Bank;
- (iii) Durchmustern der Bank auf cDNA, die die gesamten konstanten und variablen Regionen der schweren und leichten Ketten des Antikörpers codiert, und Isolieren derselben;

(iv) Insérer la cDNA, qui code les régions constantes et variables des chaînes lourdes et légères des anticorps, dans un vecteur d'expression;

(v) Transfecter une cellule hôte avec le vecteur d'expression, qui code la cDNA; et

(vi) Cultiver la cellule hôte transfectée et isoler l'anticorps souhaité.

12. Vecteur, qui est adapté pour la transfection d'une cellule hôte et qui code la cDNA des régions constantes et variables des chaînes lourdes et légères d'un anticorps de primate.

13. Lignée cellulaire eucaryotique, qui est transfectée avec la cDNA des régions constantes et variables des chaînes lourdes et légères d'un anticorps de primate.

14. Procédé d'expression de la cDNA, qui code les régions constantes et variables des chaînes lourdes et légères d'un anticorps de primate, comprenant la transfection d'une cellule hôte eucaryotique avec un vecteur ou des vecteurs, qui est adapté pour l'expression de la cDNA.

Revendications

1. Procédé pour la production d'anticorps recombinants de primate comprenant les étapes consistant à :

(i) sélectionner une lignée cellulaire dérivée de lymphocytes de primate qui est capable d'exprimer un anticorps souhaité ;

(ii) isoler l'ARN de la lignée cellulaire et séparer l'ARNm des autres ARN ainsi isolés ;

(iii) synthétiser l'ADNc de l'ARNm et insérer l'ADNc dans un vecteur de clonage ;

(iv) transformer une cellule hôte avec le vecteur contenant l'ADNc pour obtenir une banque ;

(v) cribler la banque pour trouver l'ADNc codant pour les régions constantes et variables entières des gènes des chaînes lourdes et légères d'anticorps ;

(vi) insérer l'ADNc codant pour les régions constantes ou variables entières des chaînes lourdes et légères d'anticorps dans un vecteur d'expression ;

(vii) transfecter une cellule hôte avec le vecteur d'expression contenant l'ADNc ; et

(viii) cultiver la cellule hôte transfectée et isoler l'anticorps souhaité.

2. Procédé selon la revendication 1, dans lequel le primate est un être humain.

3. Procédé selon la revendication 1, dans lequel le primate est un chimpanzé ou un singe de l'Ancien Monde.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel la lignée cellulaire est produite à partir de lymphocytes provenant d'un individu connu pour être guéri ou en rémission d'un état pathologique.

5. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel la lignée cellulaire est produite à partir de lymphocytes provenant d'un individu connu pour être infecté avec un organisme pathogène ou pour souffrir d'un cancer ou d'une maladie auto-immune, mais qui ne manifeste aucun symptôme de maladie déclarée.

6. Procédé selon les revendications 4 ou 5, dans lequel l'individu est infecté par un virus.

7. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel la lignée cellulaire est produite à partir de lymphocytes provenant d'un individu qui a été vacciné ou auquel on a inoculé un antigène et qui a présenté une réponse de l'anticorps.

8. Procédé selon l'une quelconque des revendications précédentes, dans lequel la lignée cellulaire est stabilisée ou immortalisée.

9. Procédé selon l'une quelconque des revendications 1 à 8, dans lequel la lignée cellulaire est choisie par criblage pour produire un anticorps présentant une affinité pour un antigène souhaité.

10. Procédé selon la revendication 9, dans lequel la lignée cellulaire est en outre choisie par criblage de la fonctionnalité

de l'anticorps.

11. Procédé pour la production d'un anticorps recombinant comprenant :

- 5 i) la préparation de micro-ARN à partir d'environ 1000 cellules d'hybridome ;
 ii) la génération d'une banque d'ADNc de taille choisie ;
 iii) le criblage de la banque pour trouver l'ADNc codant pour les régions constantes et variables entières des chaînes lourdes et légères d'anticorps et l'action consistant à isoler celles-ci.
10 iv) l'insertion de l'ADNc codant pour les régions constantes et variables entières des chaînes lourdes et légères d'anticorps dans un vecteur d'expression ;
 v) la transfection d'une cellule hôte avec le vecteur d'expression contenant l'ADNc ; et
 vi) la culture de la cellule hôte transfectée et l'action consistant à isoler l'anticorps souhaité.

12. Vecteur adapté pour transfecter une cellule hôte comprenant l'ADNc codant pour les régions constantes et variables entières des chaînes lourdes et légères d'anticorps de primate.

13. Lignée cellulaire eucaryote transfectée avec l'ADNc pour l'expression des régions constantes et variables entières des chaînes lourdes et légères d'anticorps de primate.

20 14. Procédé pour l'expression de l'ADNc codant pour les régions constantes et variables entières des chaînes lourdes et légères d'anticorps de primate, comprenant la transfection d'une cellule hôte eucaryote avec un vecteur ou des vecteurs adaptés à l'expression dudit ADNc.

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FIG. 1.

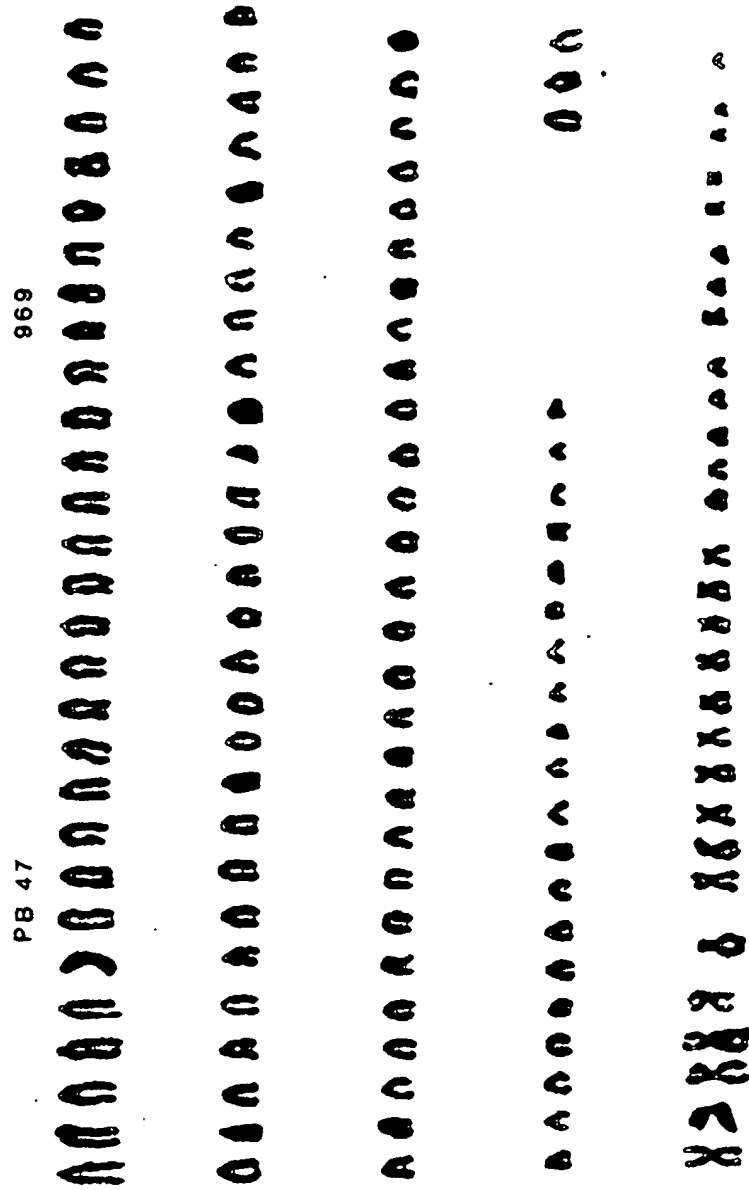


FIG. 2.

-19
M D W T W R F L

TCTAAAGAAGCCCCTGGGAGCACAGCTCATCACCATGGACTGGACCTGGAGGTTCTC 58
SIGNAL⇒

1 5

F V V A A A T G V Q S Q M Q V V Q S G A
TTTGTGGTGGCAGCAGCTACAGGTGTCCAGTCCCAGATGCAGGTGGTGCAGTCTGGGGCT 118
FR1⇒

10 15 20 25

E V K K P G S S V T V S C K A S G G T F
GAAGTAAAGAAGCCTGGGTCCTCGGTGACGGTCTCTGCAAGGCATCTGGAGGCACCTTC 178

30 31 32 33 34 35 40 45

S N Y A I S W V R Q A P G Q G L E W M G
AGCAACTATGCTATCAGCTGGGTGCGACAGGCCCTGGACAAGGGCTTGAGTGGATGGGA 238
CDR1⇒ FR2⇒

50 51 52 a 53 54 55 56 57 58 59 60 61 62 63 64 65

G I I P L F G T P T Y S Q N F Q G R V T
GGGATCATCCCTCTTTTTGGTACACCAACCTACTCACAGAACTTCCAGGGCAGAGTCACG 298
CDR2⇒ FR3⇒

70 75 80 82 a b c 83 85

I T A D K S T S T A H M E L T S L R S E
ATTACCGCGGACAAATCCACCAGCACAGCCCACATGGAGCTGACTAGCCTGAGATCTGAG 358

90 95 96 97 98 99 100 a b c d e

D T A V Y Y C A T D R Y R Q A N F D R A
GACACGGCCGTGTATTACTGTGCGACAGATCGCTACAGGCAGGCAAATTTTGACCGGGCC 418
CDR3⇒

f g h i k 101 102 105 110 115

R V G W F D P W G Q G T L V T V S S A S
CGGGTTGGCTGGTTCGACCCCTGGGGCCAGGGCACCCTGGTCAACCGTCTCCTCAGCCTCC 478
FR4⇒ C₁⇒

120 125 130 133 135

T K G P S V F P L A P S S K S T S G G T
ACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGCACA 538

140 145 150 154 156 162

A A L G C L V K D Y F P E P V T V S W N
GCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCTGTGAAC 598

165 169 171 175 180 182

S G A L T S G V H T F P A V L Q S S G L
TCAGGCGCCCTGACCAGCGGCGTGACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTC 658

185 190 195 200 203 205

Y S L S S V V T V P S S S L G T Q T Y I
TACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATC 718

FIG. 2 cont'd.

210 215 220 222 225 228 232
 C N V N H K P S N T K V D K K V E P K S
 TGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCT 778
 HINGE₄

235 240 245 250
 C D K T H T C P P C P A P E L L G G P S
 TGTGACAAAACCTCACACATCCCCACCGTGCCAGCACCTGAACTCCTGGGGGACCGTCA 838
 C₁₂

255 260 265 270
 V F L F P P K P K D T L M I S R T P E V
 GTCTTCCTCTTCCCCCAAAACCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC 898

275 280 285 290 292
 T C V V V D V S H E D P E V K F N W Y V
 ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTG 958

295 299 300 305 310 314 317
 D G V E V H N A K T K P R E E Q Y N S T
 GACGGCGTGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCAGC 1018

320 325 330 335
 Y R V V S V L T V L H Q D W L N Q K E Y
 TACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC 1078

340 345 350 355 357
 K C K V S N K A L P A P I E K T I S K A
 AAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCCATCGAGAAAACCATCTCCAAGCC 1138

360 363 365 370 375 378 381
 K G Q P R E P Q V Y T L P P S R D E L T
 AAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACC 1198
 C₁₃

385 390 395 400 402
 K N Q V S L T C L V K G F Y P S D I A V
 AAGAACCAGGTGAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG 1258

405 408 410 414 415 420 425
 E W E S N G Q P E N N Y K T T P P V L D
 GAGTGGGAGAGCAATGGGCAGCCGGAACAACCTACAAGACCACGCCTCCCGTGCTGGAC 1318

430 433 435 440 445 450
 S D G S F F L Y S K L T V D K S R W Q Q
 TCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGACAAGAGCAGGTGGCAGCAG 1378

455 460 465 470
 G N V F S C S V M H E A L H N H Y T Q K
 GGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAG 1438

475
 S L S L S P G K *
 AGCCTCTCCCTGTCTCCGGGTAAATGAGTGCGACGGCCGGCAAGCCCCGCTCCCCGGGC 1498
 TCTCGGGTTCGCACGAGGATGCTTGGCACGTACCCCGTGACATACTTCCCGGGCGCCCA 1558
 GCATGGAAATAAAGCACCCAGCGCTGCCCTGGGCCCTGCGAAAAA 1617

FIG. 3.

-19
 M A W A L L L L T
 CAAGAGGCAGCGCTCTCGGGACGTCTCCACCATGGCCTGGGCTCTGCTGCTCCTCACC 58
 SIGNAL⇒

1 5 9 11
 L L T Q D T G S W A Q S A L T Q P A S V
 CTCCTCACTCAGGACACAGGGTCCTGGGCCCAGTCTGCCCTGACTCAGCCTGCCTCCGTG 118
 FR1⇒

15 20 24 25 26 27 d e f 28
 S G S P G Q S I T I S C T G T N N D V G
 TCTGGGTCTCCTGGACAGTCGATCACCATCTCCTGCCTGGAACCAACAATGATGTTGGG 178
 CDR1⇒

29 30 31 32 33 34 35 40 45
 S Y N L V S W Y Q Q H P G K A P K I M I
 AGTTATAACCTTGTCTCCTGGTACCAGCAGCACCCAGGCAAAGCCCCAAAATCATGATT 238
 FR2⇒

50 51 52 53 54 55 56 60 65
 Y E V S K R P S G V S N R F S G S K S G
 TATGAGGTCAGTAAGCGGCCCTCAGGGGTTTCTAATCGCTTCTCTGGCTCCAAGTCTGGC 298
 CDR2⇒ FR3⇒

70 75 80 85
 N T A S L T I S G L Q A E D E A D Y Y C
 AACACGGCCTCCCTGACAATCTCTGGGCTCCAGGCTGAGGACGAGGCTGATTATTACTGC 358

89 90 91 92 93 94 95 a 96 97 100 106 a
 C S Y A G S Y T V V F G G T K L T V L
 TGCTCATATGCAGGTAGTTACACTGTGGTTTTTCGGCGGAGGGACCAAAGTACCGTCTTA 418
 CDR3⇒ FR4⇒

107 110 115 120 125
 G Q P K A A P S V T L F P P S S E E L Q
 GGTGAGCCCAAGGCTGCCCCCTCGGTCACTCTGTTCCCGCCCTCCTCTGAGGAGCTTCAA 478
 C1⇒

130 135 140 145
 A N K A T L V C L I S D F Y P G A V T V
 GCCAACAAGGCCACACTGGTGTGTCTCATAAGTGACTTCTACCCGGGAGCCGTGACAGTG 538

150 155 160 165
 A W K A D S S P V K A G V E T T T P S K
 GCCTGGAAGGCAGATAGCAGCCCCGTCAAGGCGGGAGTGGAGACCACCACACCTCCAAA 598

168 170 175 180 185
 Q S N N K Y A A S S Y L S L T P E Q W K
 CAAAGCAACAACAAGTACGCGGCCAGCAGCTATCTGAGCCTGACGCTGAGCAGTGGAAG 658

190 195 200 203 205
 S H R S Y S C Q V T H E G S T V E K T V
 TCCACAGAAGCTACAGTGCCAGGTCACGCATGAAGGGAGCACCGTGGAGAAGACAGTG 718

29

CDR1 *****

CDR2

[illegible]

FIG. 4 cont'd.

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*****
Human      60      70      80
CynoK1    CAAAGTGGGTACATCAAGGTTCAAGTGGATCTGGGACAGACTCTCACCATCAGCAGTCTGCAACCTGAAGATTCTGCA
CynoK2    A-TC-C--TC-----C-----T-----C-----G-----G-----
CynoK4    A-TC-C--TC-----C-----G-----A-----C-----G-----
CynoK5    C-GAC-----C-----G-A-C-T-T-----A-G-AA-----AG-G-GG-----GT-GG
CynoK9    C-----C-----C-----A-----C-----A-----C-----T-----
CynoK12    GA-----C-----G-----C-T-----C-----A-T-ATCC-G-G-----C-----G
CynoK14    GCCTC--A--C-GAC-----G-G-AC-T-T-----A-C-AA-----AG-G-GG-----GT-GG
CynoK15    G-----C-----C-----G-A-----A-----C-----G-----T-----
CynoK18    G-----C-----C-----G-A-----A-----C-----G-----T-----
CynoK20    GC-TC-----C-GC-----AA-----A-----G-A-----G-CG-----G-G-----C
Rabbit    T-C-T-----C-TGATC-C-----CA-----T-----G-----G-A-GGT-----CCTCA--
Mouse

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***** CDR3 *****
Human      90      95 a      100
CynoK1    ACTTACTACTGTCAACAGAGTTACAGTACCCTG ATCACCTTCGGCCAGGGACACGACTGGAGATTAA
CynoK2    GT---T-----C-C--T---GA--C- TA--GT--T---G---CAA-G---C--C---CGA
CynoK4    T-----G--A-T---TA--CT C---T---GG---CAAGG---C---CGA
CynoK5    T-----G--T-A---TG-----CT C---T---GG---CAAGG---C---CGA
CynoK9    GT---T---CATG--AGC-CTTC-GT-T-CT TGG--G-----CAAGG---A--C-G-CGA
CynoK12    T-----T---G---TG-----C- TA--GT--T---G---CAA-G---C---CGA
CynoK14    GA-----T---C-A-G---T-T-CT CGG--G-----CAAGG---A--C---CGA
CynoK15    GT---T---CATG--ATA-ACACAC-TT-CA T-----T---CC---CAA---T---C---CGA
CynoK18    T-----CG-----TA-A-T-C-TA--CA TA--GT--T---G---CAA-G---C---CGA
CynoK20    T-----T-----GA-A---TAT-CT T-----T---GG---CAAGG---C---CGA
Rabbit    T-----T---ATA-A-T---TA--CT CCG--G-----CAAGG---A--C---CGA
Mouse    T-----T---GSC---G-T---GTAGCGAT-CTG-T---GG---CGAG---CCT-TGT
CA---T-----GC-----TT-----CVAT-CT C-----G-----TGCT-----CAAG-----C-G---CGG

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513.5

[illegible][illegible]

[illegible]

FIG. 6.

	110	120	130
Human	ACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGCTTGAAATCTGGAACTGCCTCTGTGTGCTGCTGAATAAC		
Cynomolgus	G-----G-----G-T--G-----T-----		
Rabbit	GA-CCAAT--G--TA-----C--C-----A-----C-A--A--A-T-A-CA-C-----G-GCA-----A		
Mouse	G---AT-----A---A-C-----A-----CAG-----A-C-----GG-----A-C-----T-CT-----C---		
	140	150	160
Human	TTCTATCCCAGAGAGGCCCAAAGTACAGTGGAGGTGGATAACGCCCTCCCAATCGGGTAACCTCCAGGAGAGTGTACAGAGCAGGACAGC		
Cynomolgus	-----GC--A-----GGT-----A--A--A-----		
Rabbit	---CG-----AT---CAT---CC---CACC-----G---AAA---CA-A-CGG-AT-G--A-C-----ACA---CC-----AG-CC-		
Mouse	-----C-----A---CAT---T---CA-----A-T---GG-AGTGA-A-G-CAAA-GG-GT--T-A-C-----TGG--T--T-----		
	170	180	190
Human	AAGGACAGCACCTACAGCCTCAGCAGCAGCACCCCTGACCGCTGAGCAAGAGCAGACTACGAGAGAAACACAAAGTCTACGCCCTGCCGAAGTCACCCAT		
Cynomolgus	-----A-----G-----GCA-A-----C---GT-----T-----		
Rabbit	G-A---T-T-----A-----T-A-----C-----C-G--A-C-GC---GC--G--A-----G-GGT--		
Mouse	--A-----A-G-----C---T---C---G-AC--G--T--ACG---T--CAG---TA-----T--G-C--T--C---		
	200	210	
Human	CAGGCGCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTAG		
Cynomolgus	-----C-----		
Rabbit	--CAA-TC-G-----AG-GA--GTCC-----T-----T--C-----		
Mouse	A--ACATCA-CT--A---A-TGTC-----AAT-----		

FIG. 7.

Human	110	T V A A P S V F I F P P S D E Q L K S G T A S V V C L L N N F Y P R E A K V Q W L V D N A	130	140	150
Cynomolgus	A	- - - - -	- - - - -	- - - - -	- - - - -
Rabbit	D P I	- - - - -	- - - - -	- - - - -	- - - - -
Mouse	A D	- - - - -	- - - - -	- - - - -	- - - - -
Human	160	L Q S G N S Q E S V T Q Q D S K D S T Y S L S S T L T L S K A D Y E K H K L Y A C E V T H	170	180	190
Cynomolgus	- K T D	- - - - -	- - - - -	- - - - -	- - - - -
Rabbit	I - Q S G I E N	- - - - -	- - - - -	- - - - -	- - - - -
Mouse	E R Q N G V L N	- - - - -	- - - - -	- - - - -	- - - - -
Human	200	Q G L S S P V T K S F N R G E C	210		
Cynomolgus	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Rabbit	N S G - A I V Q	- - - - -	- - - - -	- - - - -	- - - - -
Mouse	K T S T	- - - - -	- - - - -	- - - - -	- - - - -

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